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Distribution of Microbial Physiologic Types in an Aquifer Contaminated by Crude Oil

B.A. Bekins, E.M. Godsy, E. Warren

U.S. Geological Survey, Menlo Park, CA 94025, USA

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Abstract

We conducted a plume-scale study of the microbial ecology in the anaerobic portion of an aquifer contaminated by crude-oil compounds. The data provide insight into the patterns of ecological succession, microbial nutrient demands, and the relative importance of free-living versus attached microbial populations. The most probable number (MPN) method was used to characterize the spatial distribution of six physiologic types: aerobes, denitrifiers, iron-reducers, heterotrophic fermenters, sulfate-reducers, and methanogens. Both free-living and attached numbers were determined over a broad cross-section of the aquifer extending horizontally from the source of the plume at a nonaqueous oil body to 66 m downgradient, and vertically from above the water table to the base of the plume below the water table. Point samples from widely spaced locations were combined with three closely spaced vertical profiles to create a map of physiologic zones for a cross-section of the plume. Although some estimates suggest that less than 1% of the subsurface microbial population can be grown in laboratory cultures, the MPN results presented here provide a comprehensive qualitative picture of the microbial ecology at the plume scale. Areas in the plume that are evolving from iron-reducing to methanogenic conditions are clearly delineated and generally occupy 25–50% of the plume thickness. Lower microbial numbers below the water table compared to the unsaturated zone suggest that nutrient limitations may be important in limiting growth in the saturated zone. Finally, the data indicate that an average of 15% of the total population is suspended.

Introduction

When residual nonaqueous hydrocarbons are present in the subsurface as contaminants, significant concentrations of reduced organic carbon continuously enter the ground water. It is well known that aquifer microbes degrade dissolved hydrocarbons under both aerobic and anaerobic conditions (for a review, see [33]). However, the ecology of the microbial populations performing the degradation reactions is poorly understood. As electron acceptors are depleted, a succession of physiologic types occurs. The spatial distribution of the physiologic types is generally inferred from aqueous concentrations of redox indicators such as dissolved

Correspondence to: B.A. Bekins; Fax: (650) 329-5590; E-mail: babekins@usgs.gov

oxygen, Fe(II), and dissolved methane. However, while these chemical species indicate that a particular reaction is occurring, their concentration distributions do not adequately demarcate the portions of the aquifer where the specific microbial processes are active. This is because the concentrations are affected not only by microbial reactions, but also by advective transport, dispersion, and inorganic reactions. Methane, for example, can be transported conservatively long distances, making it difficult to determine the exact location and extent of the methanogenic zone. Moreover, several workers have suggested that the anaerobic cores of plumes are extremely narrow in vertical extent (e.g., [9, 31]) and that pore water samples from wells with large screens may be sampling ground water from two or more redox zones.

A microbial population capable of using a specific electron acceptor presumably expands, as chemical conditions become optimal, and dies back as the electron acceptor is depleted. However, models that have attempted to reconcile theoretical growth formulations with populations observed in the aquifer have encountered difficulties (e.g., [3, 8, 13]). Aspects of the microbial growth model formulation that need improvement include population changes associated with transitions in redox conditions and the predicted growth rate of a given population. Essaid et al. [13] documented model sensitivity to parameters controlling the transition from iron-reducing to methanogenic conditions. In particular, the coexistence of the two populations gave a better fit to chemical and microbial data than a sharp transition from one type to another. For a given physiologic type, the existing growth models also predict higher microbial numbers than are found in the field. One possible process that would act to limit the net growth is microbial transport. Existing field observations suggest that free-living populations increase within contaminant plumes [17, 19], and laboratory results show that microorganisms are capable of active processes that promote detachment and attachment to sediment (e.g., [30]). Although the literature contains several bacterial transport models, these models do not account for active attachment and detachment [25].

The goal of this work was to qualitatively delineate the distribution of microbial redox zones in a plume of crude-oil contaminants by examining the distribution of microbial physiologic types. Characterizing these microbial zones at the plume scale provides insight into the patterns of ecological succession and the role of trace nutrients in controlling population growth. Using the most probable number (MPN) method, the spatial distribution was characterized for six physiologic types of microorganisms: aerobes, denitrifiers, iron-reducers, heterotrophic fermenters, sulfate-reducers, and methanogens. The samples were collected over a vertical cross-section of the aquifer oriented parallel to the direction of ground-water flow, corresponding to the longitudinal axis of the plume. We concentrated on the anaerobic portion of the plume because anaerobic processes probably account for the majority of the hydrocarbon degradation at this site [13]. The relative aqueous solubilities of hydrocarbons and oxygen together with the stoichiometric ratio of aerobic degradation result in complete consumption of oxygen as ground water contacts the nonaqueous phase. For this reason, anaerobic processes are estimated to be important at most petroleum hydrocarbon contaminated sites where a nonaqueous phase is present. By performing MPN estimates of both suspended and attached populations, we also examine whether microbial transport may be important in the plume. The observations presented here form a basis for designing controlled laboratory experiments and field studies in support of better model formulations for growth and ecological succession of the active degrading organisms in contaminated aquifers.

Site Description

The study aquifer, located near Bemidji, Minnesota, is a surficial formation of pitted and dissected glacial outwash sediments (Fig. 1). It was contaminated with crude oil when a pipeline ruptured in August 1979, spilling approximately 10,000 barrels of oil on the land surface. After clean-up operations by the pipeline company, approximately 30% of the spilled oil or 5×10^5 L (3,200 barrels) remained and subsequently infiltrated into the aquifer, forming two main bodies of oil floating on the water table [21]. A set of companion papers [2, 4, 12] described the geochemistry of the contaminant plume emanating from the larger oil body (north pool in Fig. 1). The uncontaminated ground water is aerobic with dissolved oxygen ranging from 8 to 9 mg/L. It contains some dissolved organic carbon (2.8 mg/L as C), and low levels of nitrate (44.8 μg/L as nitrogen) and sulfate (2.9 mg/L) [4].

The temporal evolution of geochemical conditions in the subsurface plume was described by Baedecker et al. [2]. Starting in 1984, concentrations of both reduced iron and methane began to increase in the anoxic zone immediately downgradient of the oil body. From 1986 to 1989, profiles of dissolved organic compounds indicated that the plume

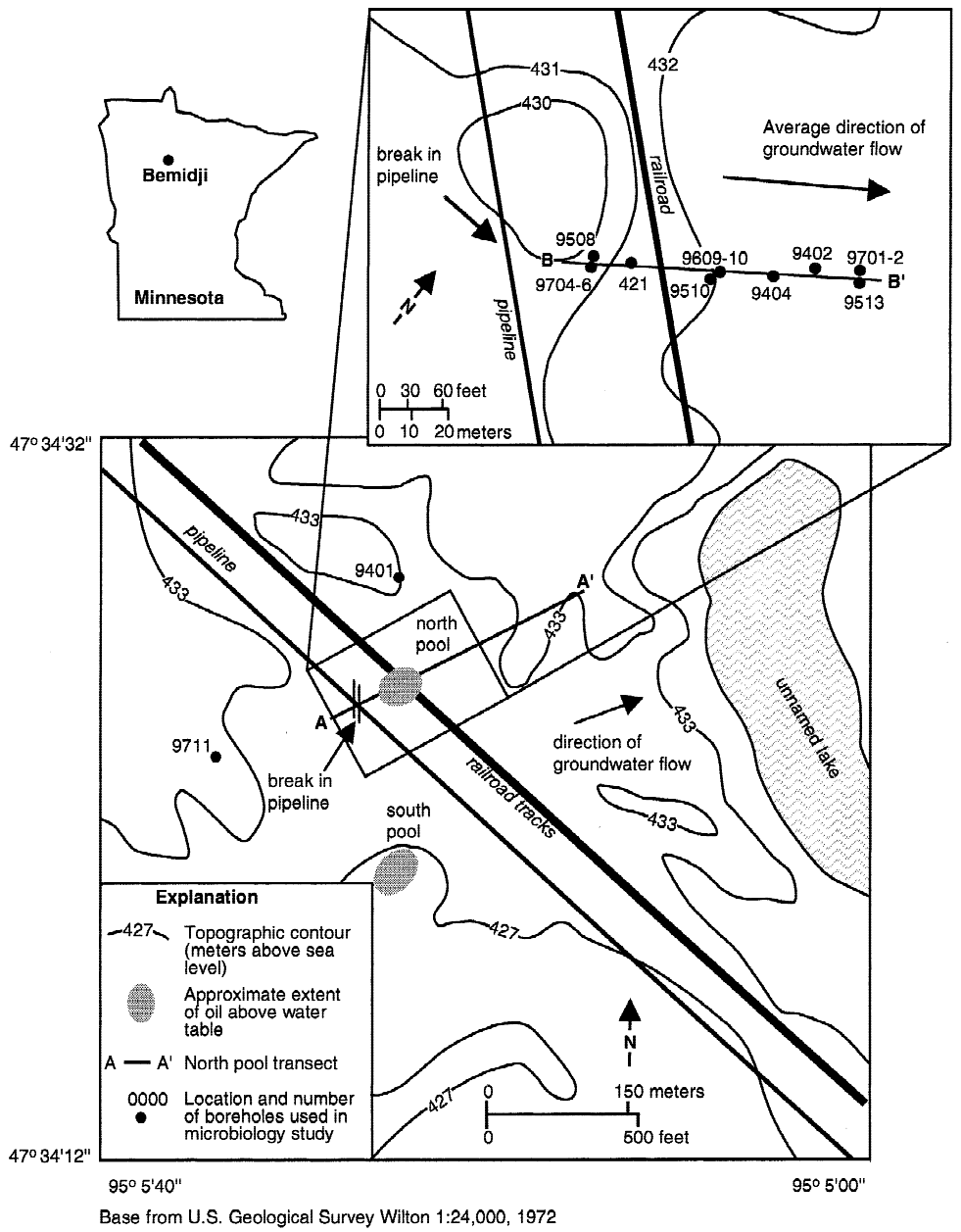

Fig. 1. Map of the Bemidji research site showing the oil pipeline, the two main nonaqueous oil bodies known as the north and south pools, and the microbiology core locations (background sites are on large map, plume sites on small map). The first two digits of the core numbers denote the year of collection.

reached a quasi-steady state in which dissolution from the nonaqueous oil was approximately balanced by biotransformation. On the basis of the pore-water chemistry, Baedecker et al. [2] divided the aquifer in the vicinity of the oil body into anoxic, transition, and background zones. Within the anoxic portion of the plume, hydrocarbons are oxidized predominantly by iron reduction [22] and methanogenesis [2, 4]. Figures 2A and B contain contour plots of dissolved oxygen (DO) and dissolved volatile organic carbon (VOC) measured in 1992 along the axis of the north pool plume [1, 10]. These plots allowed precise siting of cores collected from five locations to characterize the microbial population in the anaerobic portion of the plume. Note that the anaerobic core of the plume attains a minimum vertical thickness of about 1 m at the downgradient edge of the nonaqueous oil body. Essaid et al. [13] showed that restricted recharge through the nonaqueous oil body causes the observed upwelling of uncontaminated, oxygenated water from below the plume.

Materials and Methods

Core Collection

A total of 11 cores were collected during the summers of 1994–1997. The 47-mm diameter cores were collected in clear polycar-

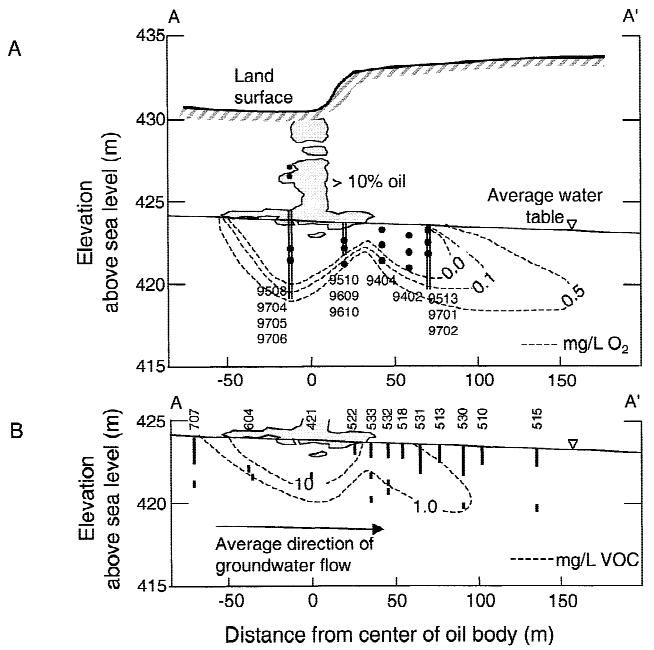

Fig. 2. Cross-sections of 1992 concentration distributions in the contaminant plume below the north pool oil body along the line A–A′ of Fig. 1. The area with greater than 10% oil saturation determined by Dillard et al. [11] is also shown. (A) Dissolved oxygen distribution modified from Cozzarelli et al. [10] together with the microbial sample locations. The filled black circles mark the 14 saturated zone and two unsaturated zone point sample locations. The double lines mark the locations of detailed vertical profiles. (B) Total dissolved volatile organic carbon from Baedecker et al. [1]. Screened intervals and ID numbers of monitoring wells are also shown.

bonate liners prerinsed with methanol and deionized water. Using a hollow-stem auger, a borehole was drilled to the top of the desired sample interval. Next, an 8-foot piston core barrel was driven beneath the augers through the target sample interval. The bottom of the core was then frozen *in situ* before retrieval thus preserving the position of the ground water with respect to the cored sediments [26]. After retrieval, the cores were removed from the core barrel and immediately sealed with caps at the top and bottom.

Microbial Sampling

To facilitate collection of water samples, the upper end of the core was capped with a pressure-fit stopper coupled to a gas line and pressurized at less than 12 kPa with oxygen-free nitrogen gas. A small hole was drilled in the sediment core liner at the desired sampling depth. The luer fitting on a sterile 10 mL glass syringe was then inserted and the syringe was filled with water. A sterile 23 gauge needle was attached and the water sample was injected into a sterile 30 mL glass serum bottle sealed with a butyl rubber stopper under an oxygen-free nitrogen atmosphere. The water samples were stored for not more than 4 hr at 20°C before inoculation of the growth media.

Immediately after the water sample was removed, the core was cut with a large tubing cutter, exposing the aquifer material at the base of the water sample interval. Under a flow of oxygen-free nitrogen gas, the first few centimeters of the core material at the cut were removed with a sterile spatula, exposing an uncontaminated surface. Approximately 10 g of sediment from the center of the core was then added to a 25 × 142 mm anaerobic isolation roll streak tube (Bellco Glass Inc., Vineland, NJ. Note: Any use of trade, product, or firm names in this paper is for descriptive purposes only and does not imply endorsement by the U.S. government) filled with 20.0 mL of mineral salts solution. The mineral salts were prepared as follows (per liter): 0.75 g of KH_2PO_4; 0.89 g of K_2HPO_4; 0.36 g of $MgCl_2 \cdot 6H_2O$; 0.9 g of NH_4Cl; 9.0 mL of trace metal solution [36]; 5.0 mL of vitamin solution [34]; and 10 mg of Tween 80 (a nonionic surfactant added to remove microbes from the sediment [35]). The pH was adjusted to 7.0, and the solution was then boiled, cooled, and dispensed under a stream of oxygen-free nitrogen gas. The solution was sterilized at 121°C (100 kPa) for 15 min. Prior to use, a sterile solution of amorphous FeS [7] was added as a reducing agent to final concentration of 1% by volume. Oxygen-free nitrogen gas was allowed to flow over the surface of the mineral salts solution as the sediment sample was added. The tube was then sealed, mixed well, and allowed to stand for 2 hr to allow penetration of Tween 80 into the sample. The tubes were then opened and sonicated (10 W for 30 s) to dislodge the bacteria into the mineral salts using a Branson Sonifer, Model 200, with a microtip (Branson Ultrasonics Corporation, Danbury, CT) under a flow of sterile oxygen-free nitrogen gas. The sediment samples in mineral salts were stored for not more than 4 hr at 20°C before inoculation into growth media. Throughout the text, we refer to the numbers from the sediment samples as "attached numbers." Because of the sampling methodology, realistically these numbers include a small proportion of the suspended population that remains in the residual water on the sediments.

Microbial Population Determinations

Microbial populations in both water and sediment samples were determined using a five-tube MPN analysis. The MPNs are based on a statistical analysis of five replicate dilutions. As part of the replicate analysis, 95% confidence intervals are also obtained. For five-tube replicates, the upper end of the 95% confidence interval corresponds to about three times the MPN and the lower end to one-third of the MPN. On a $\log_{10}$ scale this is plus or minus one-half log unit [24]. Samples were serially diluted by orders of magnitude into dilution mineral salts solutions that were prereduced and anaerobically sterilized (PRAS) as described by [20]. Aliquots of the dilutions were inoculated into six different media, designed to promote growth and enumeration of aerobic and anaerobic heterotrophic, denitrifying, iron-reducing, sulfate-re-

ducing, and methanogenic microorganisms. Negative controls contained the same media, but were not inoculated. Bacteria capable of aerobic heterotrophic growth were enumerated using Standard Methods Broth (BBL Microbiology Systems, Cockeysville, MD). Tubes with visible growth after incubation at room temperature for 1 week were scored positive. Microorganisms capable of anaerobic heterotrophic growth or fermentation were enumerated using PRAS prepared Schaedler's Broth (Difco, Detroit, MI). Tubes with turbid growth or clumps of particulates after incubation at room temperature for 1 week were scored positive. Those bacteria capable of denitrification were enumerated using the media described by Stanier et al. [32]. The denitrifying medium was dispensed into 16 × 100 mm screw-cap test tubes that contained an inverted 6 × 50 mm test tube. The presence of denitrifying bacteria was determined by the presence of nitrogen gas in the inverted tube and visible growth in the medium after incubation for 2 weeks at room temperature.

Iron-reducing bacteria were enumerated using PRAS prepared medium consisting of (per liter) 2.5 g of sodium acetate · $3H_2O$; 2.5 g of $NaHCO_3$; 0.1 g of $CaCl_2 \cdot 2H_2O$; 0.1 g of KCl; 1.5 g of NH_4Cl; 1.0 g of KH_2PO_4; 0.1 g of $MgCl_2 \cdot 6H_2O$; 0.1 g of $MgSO_4 \cdot 7H_2O$; 9 mL of trace metal solution; 5 mL of vitamin solution; and 200 mL of 0.5 M Fe^{3+} [23]. The pH was adjusted to 7.0. The medium was dispensed into 10 mL serum bottles and autoclaved at 121°C (100 kPa) for 15 min. After inoculation, the serum bottles were aseptically pressurized with a 70:30 mix of H_2:CO_2 to 140 kPa. The serum bottles were allowed to incubate for a minimum of 6 weeks at room temperature. After incubation, 1.0 mL of a solution containing a 1:2 ratio of 2 g/L bipyridine and 350 g/L sodium acetate was injected into each of the serum bottles to determine the presence of reduced iron (Fe^{2+}). Under these conditions, the presence of greater than 2 mg/L of reduced iron caused the clear solution to turn pink to red and was scored as a positive. The negative control was also treated with bipyridine and the resulting pale pink color was used as a no-growth standard.

Medium for the enumeration of sulfate-reducing bacteria consisted of PRAS mineral salts medium prepared as follows (per liter): 3.0 g of Na_2SO_4; 0.2 g of KH_2PO_4; 0.3 g of NH_4Cl; 0.5 g of KCl; 0.15 g of $CaCl_2 \cdot 2H_2O$; 1.0 g of NaCl; 0.4 g of $MgCl_2 \cdot 6H_2O$; 2.8 g of sodium acetate · $3H_2O$; 9.0 mL of trace metals; and 4.0 mL of vitamin solution. The pH was adjusted to 7.2. The medium was dispensed into 10 mL serum bottles and autoclaved at 121°C (100 kPa) for 15 min. Just before use, 0.1 mL of sterile, CO_2-saturated $NaHCO_3$ solution (84 g/L) was added to each of the serum bottles. After inoculation, the serum bottles were aseptically pressurized with a 70:30 mix of H_2:CO_2 to 140 kPa. The presence of sulfide, and hence sulfate-reducing bacteria, was determined by dipping lead acetate paper (Fisher Scientific, Pittsburgh, PA) into the medium after 0.5 mL of 1 M NaOH was added to ensure that all HS^- was converted to S^{2-}. Any darkening of the paper, indicating a S^{2-} concentration greater than 0.25 mg/L, was scored as positive.

Microorganisms capable of methane production were enumerated on PRAS dilution mineral salts medium. Acetoclastic and formate-utilizing organisms were enumerated under a nitrogen and CO_2 atmosphere with the addition of 2.5 g of sodium acetate · $3H_2O$ or 2.5 g sodium formate per liter, respectively. Hydrogen oxidizers were enumerated by aseptically pressurizing the serum bottles after inoculation with a 70:30 mix of H_2:CO_2 to 140 kPa. Total numbers of methanogens were enumerated with mineral salts media containing all three substrates. The serum bottles were allowed to incubate for a minimum of 6 weeks at room temperature. The presence of methane in the head space was determined by gas chromatography/flame ionization detection analysis [16]. Negative controls were used to establish a background methane concentration.

Results

Closely Spaced Vertical Profiles in the Saturated Zone

Figure 3 shows plots of the sediment microbial numbers for three vertical profiles from the locations shown in Fig. 2. The populations of aerobes and iron-reducers vary by two to three orders of magnitude over short vertical distances of 10–20 cm. The methanogen numbers shown are the sum of the separate numbers obtained for hydrogen-, formate-, and acetate-utilizers. The peak populations of the summed methanogens are only 10^2/g-sediment, but these contrast sharply with nondetectable background numbers. These results are similar to those of Godsy et al. [17], who reported a 100-fold increase in methanogens in a plume of dissolved creosote compounds compared to an uncontaminated background location. Like the aerobes and iron-reducers, the methanogenic populations vary vertically on a scale of 10–20 cm.

In all three profiles it appears that peaks in iron-reducer numbers alternate with those of methanogens. A Pearson correlation analysis between the two populations using the log numbers for the three profiles combined gives an inverse correlation of −0.62 ($p < 0.0007$; 24 samples). Although the MPN method is not reliable for determining absolute numbers, the consistent pattern in relative numbers among the three profiles is striking. The profile in Fig. 3A from below the oil (located 12 m upgradient from the center of the oil body) shows that iron-reducers are dominant at three levels within the saturated zone: 424.0 m, 421.8 m, and 419.0 m above sea level. At these same levels, there are lower numbers of fermenters, methanogens, aerobes, and sulfate-reducers. Conversely, locations with lower numbers of iron-reducers found at levels 424.5 m and 420.0–421.5 m correspond to higher numbers of fermenters, methanogens, aerobes, and sulfate-reducers.

The profile shown in Fig. 3B from the upwelling area (located 20 m downgradient from the center of the oil body)

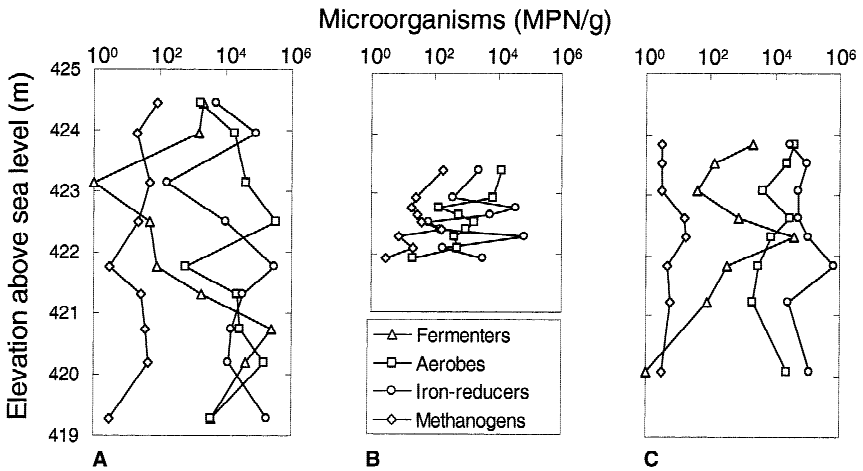

Fig. 3. Distributions of physiologic types attached to the sediments for the three vertical profiles shown in Fig. 2 through the plume's anoxic core. Profiles are located (A) −12 m, (B) 20 m, and (C) 65 m from the center of the oil body. Five physiologic types are displayed for profiles A and C, whereas only three types were determined for profile B. The 95% confidence intervals for the five-tube MPN method are roughly plus or minus one-half log unit. In all of the profiles, there is a striking inverse correlation between methanogens and iron-reducers: low methanogen numbers are associated with higher numbers of iron-reducers, and low iron-reducer numbers correlate with higher numbers of methanogens, aerobes, sulfate-reducers, or fermenters.

shows the smallest scale oscillations between methanogenic and iron-reducing conditions. Two elevations in the aquifer, 422.7 m and 422.3 m, have peak iron-reducing numbers together with relatively low numbers of aerobes and methanogens. At two other levels, peak values of aerobes and methanogens are found together with lower numbers of iron-reducers. One level is located within the oil body at 423.4 m and the other is at 422.3–422.6 m in the core of the plume. A third level exhibiting this pattern is possibly also present at 422.1 m. Thus, this profile has methanogenic zones alternating with iron-reducing zones on a scale of only 25 cm.

The core sample collected 65 m downgradient (Fig. 3C) shows signs of being less evolved toward methanogenic conditions than the profiles under the oil. Peak aerobe numbers are found at the top and base of the profile where aerobic conditions exist at the edge of the plume. A third peak in aerobes also exists between 422 and 423 m above sea level, where methanogens, fermenters, and sulfate-reducers increase and iron-reducers decline. The maximum number of methanogens found is almost 10 times lower than at the sites below the oil. Moreover, the number of culturable iron-reducers still present in the evolving methanogenic zone exceeds 10^4/g, compared to values as low as 10^2/g in methanogenic zones closer to the oil. Thus, the data suggest that, compared to sites below the oil, this location is in the early stages of switching from iron-reducing to methanogenic conditions.

In summary, the closely spaced profiles illustrate a pattern of methanogenic zones alternating with iron-reducing zones on scales of 25 cm to 1 m. However, in most locations, active iron-reducers and methanogens are both present. Therefore, reactions expected under both methanogenic and iron-reducing conditions may be occurring simultaneously throughout most of the profile.

Spatial Distribution of Physiologic Types in the Saturated Zone

Figure 4 shows an interpretation of physiologic zones inferred from the distribution of microorganisms found attached to the sediments. These zones are based on MPN data from the three vertical profiles (Fig. 3) and 14 broadly spaced point samples collected from below the water table (Fig. 2A). An area is classified as methanogenic if more than 10/g total methanogens were present on the sediments and iron-reducer numbers were relatively low. When iron-reducer populations achieved maximum values and attached methanogens declined, the location was classified as iron-reducing. Finally, if only aerobes and fermenters were found, the location was classified as aerobic.

We note that the presence of culturable organisms of a given physiologic type does not prove that the associated process is active in the aquifer. However, we feel that delineating physiologic zones on the basis of the attached populations is appropriate for several reasons. First, the vertical

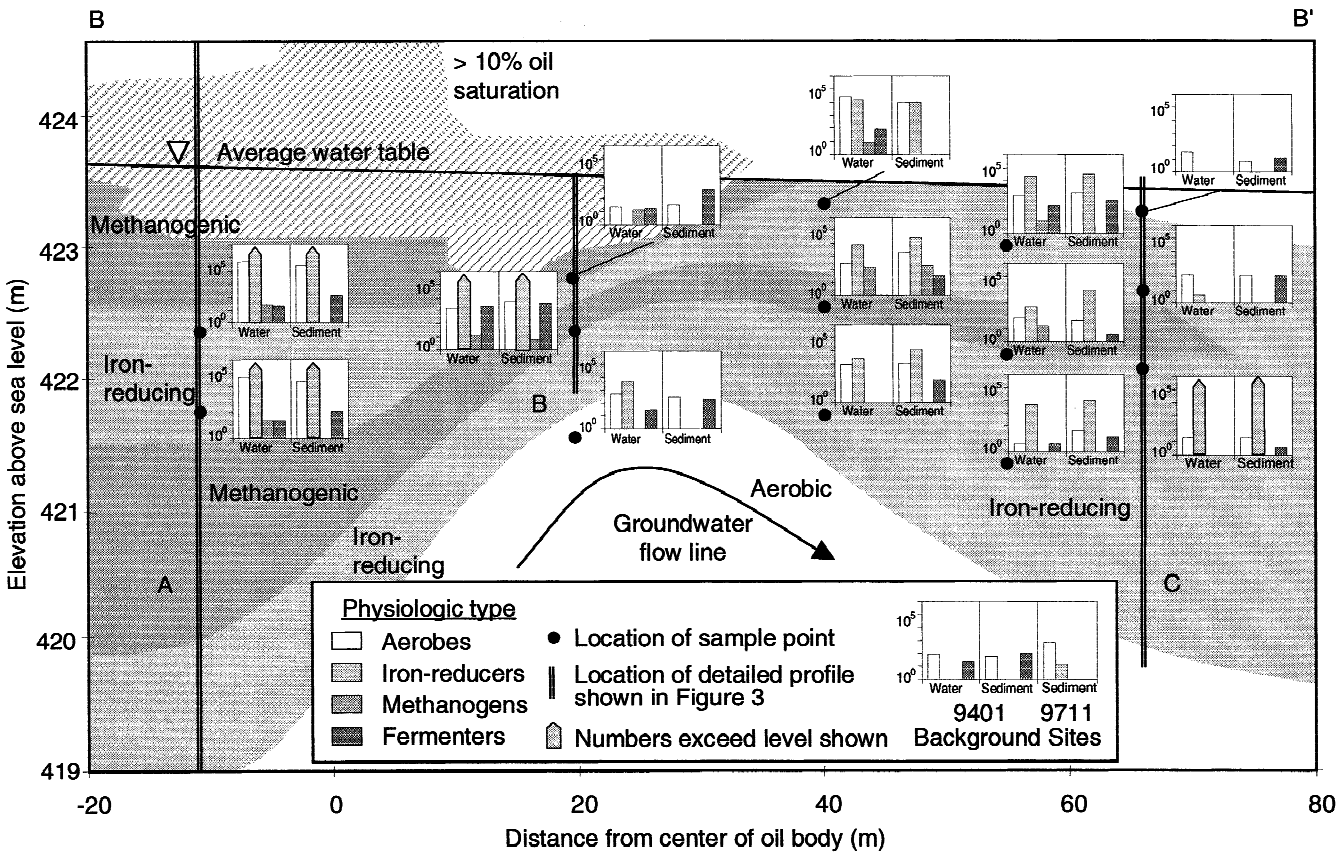

Fig. 4. Expanded cross-section of the anaerobic portion of the aquifer summarizing the inferred distribution of physiologic types. The superimposed bar graphs show numbers for the marked sample locations of four physiologic types: aerobes, iron-reducers, methanogens, and heterotrophic fermenters. The vertical axes are on a log scale ranging from 1 to 10^6. For each sample, the left-hand side labeled "water" shows suspended numbers per mL of drained water, while the right-hand chart labeled "sediment" shows the attached numbers per gram dry weight of sediments. Data for two uncontaminated background sites are shown in the legend. The 95% confidence intervals for all bar charts are roughly plus or minus one-half log unit. Dashed vertical lines indicate the locations of detailed vertical profiles presented in Fig. 3. Note that the top of the plot lies 5–8 m below the land surface.

profile data show a significant inverse correlation between the attached methanogens and iron-reducers, indicating that these attached physiologic types exhibit spatial zonation in the aquifer. Second, several researchers have noted that attachment may convey an advantage by facilitating exchange among members of a consortium [14] or access to solid-phase metals [27] or other nutrients [29]. Finally, with few exceptions, the distribution of the suspended population in the aquifer is similar to that of the attached population (Figs. 5 and 6).

The bar charts superimposed on the zone map show the estimated suspended and attached microbial numbers for the 14 point samples. Data for two background sites are also shown in the legend. Although six physiologic types were measured, the results for only the four major types are displayed in the bar charts: aerobes, iron-reducers, methano-

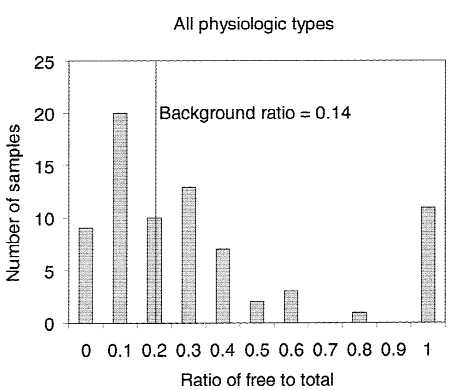

Fig. 5. Histogram of the ratio of numbers found in the water to total population at a sample location. Data from the widely spaced samples and the detailed profiles for all physiologic types are combined to yield a total of 97 samples.

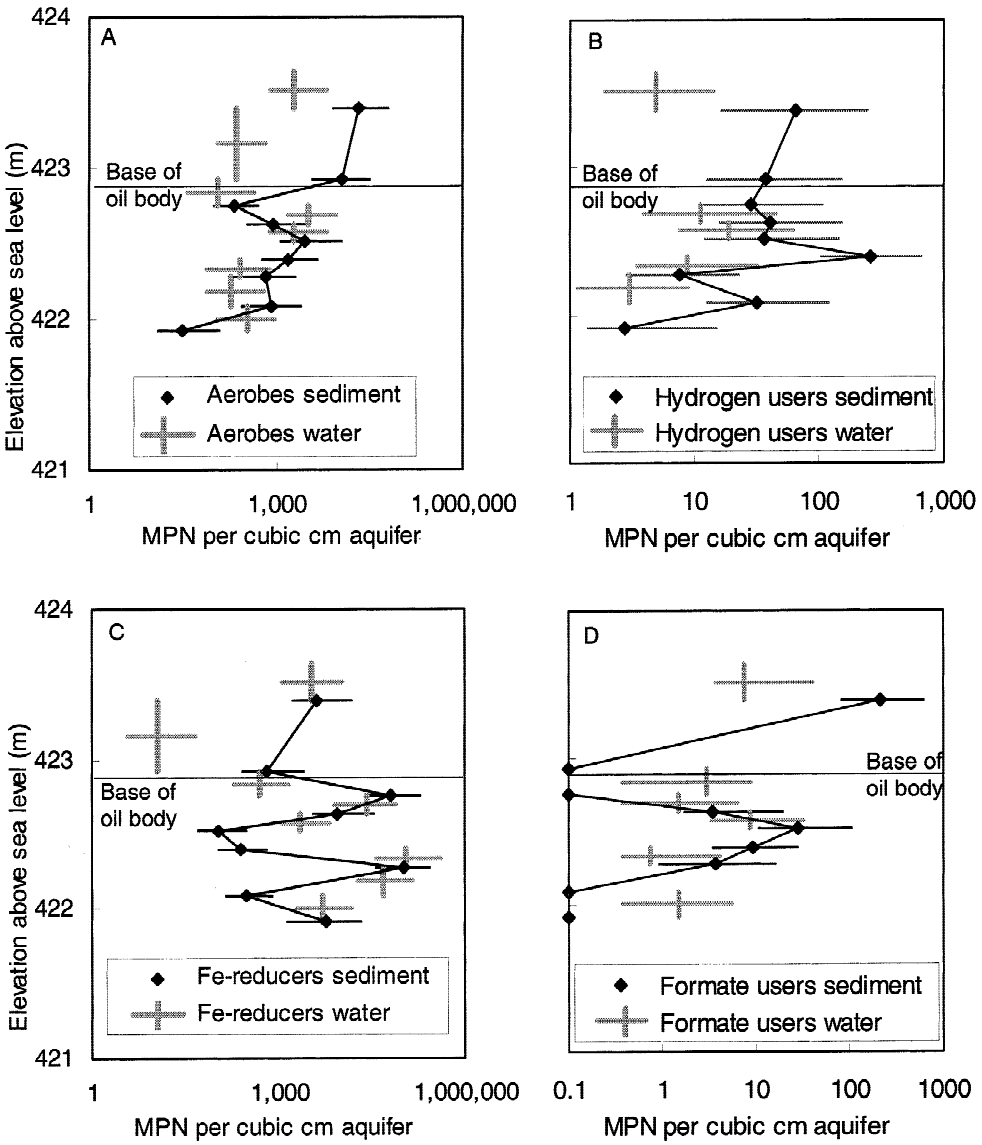

Fig. 6. Comparison of sediment and water populations of four physiologic types for a detailed profile located 20 m downgradient from the center of the oil body (Core 9610 in Fig. 2). (A) aerobes; (B) hydrogen-utilizing methanogens; (C) iron-reducers; and (D) formate-utilizing methanogens. For both sediment and water, the units are MPN per cm^3 of aquifer. Water values are shown as vertical bars spanning the drained interval with error bars in the center of the interval. The values for the sediments are plotted with error bars where samples were obtained between drained intervals. The location of the base of the oil body is also shown.

gens, and heterotrophic fermenters. Denitrifier and sulfate-reducer numbers are given in Table 1. At the background sites, low numbers of aerobes and heterotrophic fermenters (10^2/g and /mL) were present both in the water and attached to the sediments. These numbers are similar to those found at other pristine locations [15]. A few sulfate-reducers (<10/g) and iron-reducers were also present, and denitrifiers were detected in the water only (Table 1).

The microbial populations within the plume are consistent with the known geochemical evolution of the site. Iron-reducers form the largest group in the anoxic core of the plume with culturable numbers usually ranging over 10^4–10^6/g and 10^1–10^6/mL. These numbers are generally consistent with values from other contaminated sites [15]; however, they contrast markedly with the pristine background sites where iron-reducers were present in low numbers. At four locations near the fringes of the plume, iron-reducers were not detected on the sediments (see top and base samples at 20 m; and top two samples at 65 m downgradient from the center of the oil body, Fig. 4). The overall picture is that high numbers of iron-reducers are found both in the water and on the sediments within the anoxic core of the plume. Outside this zone, iron-reducers quickly decline in number and are sometimes found only in the water.

Methanogens were found both in the water and on the sediments at two locations in the core of the plume (see middle samples at 20 m and 40 m). Six additional locations, around the fringes of the methanogenic zone, had methanogens only in the water. The aerobes form the second largest population group in the plume and were detected in every sediment and water sample. Numbers of culturable aerobes were 100–1,000 times greater than at the uncon-

Table 1. Denitrifier and sulfate-reducer numbers

Sample	Distance	Elevation	SO_4-reducers	Denitrifiers
9508-1s	−10.6	422.3	<0.3	<0.3
9508-1w	−10.6	422.3	0.60	0.60
9508-2s	−10.6	421.7	1.76	0.78
9508-2w	−10.6	421.7	2.38	0.90
9510-11s	19.8	422.7	<0.3	<0.3
9510-11w	19.8	422.7	0.60	<0.3
9510-21s	19.8	422.3	1.88	1.41
9510-21w	19.8	422.3	2.11	<0.3
9510-22s	19.8	421.5	<0.3	<0.3
9510-22w	19.8	421.5	<0.3	<0.3
9402-1s	55.3	423.0	1.53	<0.3
9402-1w	55.3	423.0	0.60	0.90
9402-2s	55.3	422.1	1.32	<0.3
9402-2w	55.3	422.1	0.60	<0.3
9402-3s	55.3	421.3	<0.3	0.60
9402-3w	55.3	421.3	<0.3	<0.3
9404-1s	40.4	423.3	<0.3	<0.3
9404-1w	40.4	423.3	<0.3	1.26
9404-2s	40.4	422.5	<0.3	0.95
9404-2w	40.4	422.5	1.40	<0.3
9404-3s	40.4	421.7	<0.3	<0.3
9404-3w	40.4	421.7	<0.3	<0.3
9404-4w	40.4	421.3	<0.3	<0.3
9513-1s	66.1	423.3	<0.3	<0.3
9513-1w	66.1	423.3	<0.3	<0.3
9513-2s	66.1	422.7	1.90	<0.3
9513-2w	66.1	422.7	0.60	<0.3
9513-3s	66.1	422.1	0.90	<0.3
9513-3w	66.1	422.1	1.08	<0.3
9711-1s	backgrnd		<0.3	<0.3
9401-1s	backgrnd		0.60	<0.3
9401-1w	backgrnd		0.30	0.30

taminated background sites, even though all but two of the samples were collected from the area with no detectable dissolved oxygen.

On the basis of Figs. 3 and 4, there are apparent relationships among the aerobes, methanogens, and iron-reducers. These trends are difficult to assess rigorously, because aerobes also increase at the fringes of the anoxic zone where no methanogens and few iron-reducers are found. In order to statistically evaluate population trends in the anoxic portion of the aquifer, we formed a composite data set that included all of the sediment samples from both the closely spaced profiles and the broadly spaced samples. From these data, we then excluded samples with no detectable iron-reducers on the assumption that these are not representative of the anerobic population. Thus, we had a total of 42 samples from which six samples were omitted: two background locations and four from the fringes of the plume (Fig. 4). A correlation analysis on the restricted data set from the anoxic zone gave a positive correlation between the methanogens and aerobes of 0.32 ($p < 0.05$; 36 samples). The tendency of these organisms to increase with the methanogens suggests they are part of a consortium growing under anerobic conditions, as opposed to a dormant remnant from when conditions were aerobic. Presumably they can metabolize organic substrates by either a fermentative or aerobic pathway. The inverse correlation between iron-reducers and methanogens found in the closely spaced vertical profiles is also apparent in this comprehensive anoxic zone data set, yielding a correlation coefficient of −0.59 ($p < 0.00007$; 36 samples).

The numbers of culturable fermenters ranged over 10–1,000/g or /mL and were comparable to background numbers except at one locatioin (see middle sample at 20 m), where they were ten times higher than background. The fermenters are generally lower in number than the aerobes, and there does not appear to be a systematic variation between the two populations. In the vertical profiles (Fig. 3), both populations peak in the methanogenic zones, but the peaks are usually offset from each other. In the background data, the aerobe and fermenter numbers are indistinguishable within the estimated errors. Because the uncontaminated aquifer is aerobic, the similar numbers may reflect a single population of facultative organisms presently living aerobically and utilizing the native DOC. When cultured, these organisms may grow in our MPN tubes either aerobically or as heterotrophic fermenters. A Pearson correlation analysis of all the data provides no significant result for either correlation or complete independence of the aerobes and fermenters. It appears that the populations cultured in the aerobic media and the fermenting media exhibit some overlap in the background, but also some noteworthy differences (particularly in the anoxic zone).

Denitrifiers and sulfate-reducers were present in low numbers (Table 1), presumably because of the low nitrate and sulfate concentrations found in the ground water at the site [4]. Sulfate-reducers were found at 12 out of the 14 locations. The highest numbers were only 10^2/g and /mL, but these are over ten times the background values. The small but significant population increase is consistent with a decrease in dissolved sulfate from a background value of 2.9 mg/L to below detection in the core of the plume [4]. Denitrifiers were present in 4 out of the 14 locations in minor numbers that are less than 10 times above the background.

The overall pattern of the microbial distribution matches that of the DO and VOC contour plots (Fig. 2). The boundary of the iron-reducing zone at the base of the plume is similar to the 0.5 mg/L DO contour (Fig. 2A). This is clear

from the lack of iron-reducers found at the base of the upwelling area (at 20 m downgradient). Thus, there is a vertical narrowing of the anaerobic population in this area compared to the thicker profiles upgradient and downgradient. The microbial population distribution also has a bimodal character in which methanogenic zones have evolved in two places: one within the area of the aquifer that contains nonaqueous oil, and a second below the oil in the laterally migrating plume. In the laterally migrating plume there is a classic pattern of a methanogenic core progressing to iron-reducing and then aerobic conditions. The thickness of the methanogenic zone seems to scale with the overall vertical thickness of the plume. In the central upwelling area, where the plume is about 1 m thick, the methanogenic zone spans 0.25–0.5 m. In contrast, where the plume is 4 m thick, the methanogenic zone spans 1–2 m. In each case, the methanogenic zone covers 25–50% of the dissolved plume.

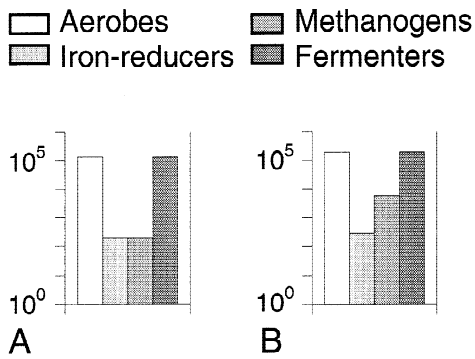

Fig. 7. Distributions of physiologic types in the unsaturated zone for the two locations marked in Fig. 2. Oil saturation of samples was (A) less than 10%; (B) greater than 10%. The results indicate that methanogenic activity at some locations in the unsaturated zone is significantly greater than in the saturated zone.

Suspended versus Attached Populations

The data provide some insight into the relation between suspended and attached populations of each physiologic type of microorganism. Both suspended and attached numbers were available for 23 (15 widely spaced and 8 closely spaced) sample points. Using all the values for the important physiologic types (aerobes, iron-reducers, methanogens, and fermenters) yielded a total of 76 pairs of suspended and attached numbers. To facilitate comparisons it was necessary to convert to number per unit volume of aquifer material (MPN/cm^3). Thus, the sediment numbers were multiplied by the average bulk density (1.64 g/cm^3), and the water numbers were multiplied by the average porosity (0.38). The resulting values were added to obtain a total per cubic centimeter of aquifer material and the ratios of suspended to total numbers were computed. A histogram of the ratios (Fig. 5) exhibits a bimodal distribution. In over 80% of the samples the majority of the population is attached to the sediments. In 11 cases the population is entirely suspended. Eight of these can be seen in Fig. 4. These are methanogens in two samples 12 m upgradient from the center of the oil between the levels of 421.5 and 422.5 m above sea level, and in the top samples at 20, 40 (there are also suspended fermenters at this site), and 55 m downgradient from the center of the oil body. In the sample 20 m from the oil body center, iron-reducers were found only in the water just below the base of the interpreted iron-reducing zone.

To further examine this issue, both suspended and attached populations for a closely spaced profile below the oil body are illustrated in Figs. 6A–D. The core was sampled continuously, such that for each drained interval, a sediment sample was taken at both the top and the base of the drained interval with interval lengths ranging from 15 to 25 cm. For all of the physioloigc types, the population in the water is generally comparable to the highest adjacent value found on the sediments. In some cases the highest adjacent sediment value is located at the top of the drained interval, while in other cases it lies at the base. There are a few exceptions to this trend in the intervals 422.8–422.9 m and 421.9–422.1 m above sea level, where formate-utilizing methanogens are detected in the water but not on the adjacent sediments (Fig. 6D). Within the oil body, below the water table, the populations of all four types are 10–100 times lower in the water than on the sediments. Such a low ratio of free to attached numbers does not occur in any other samples in this profile. These data could be consistent with the toxicity response observed by Bitton and Freihofer [5], in which attached populations gain protectioin from high concentrations of toxic compounds present near the nonaqueous source. Alternatively, the hydrophobicity of oil coated sediments could be a factor.

Unsaturated Zone

Figures 7A and B show the populations found in the two sediment samples from the unsaturated zone: one from a location with greater than 10% oil saturation and the other from a nearby location with less than 10% oil. Both samples contained a relatively large and diverse population of anaer-

obes. In particular, they contained significant numbers of methanogens: 1–30 times the highest value from the saturated zone. Numbers of sulfate-reducers and denitrifiers at these two sites are also higher than at any location in the saturated zone. In contrast, iron-reducers are present but their numbers are only about 10^2/g, which is comparable to the lowest numbers from the saturated zone (Fig. 3). In both cases, low numbers of iron-reducers are associated with increased numbers of methanogens, suggesting that anaerobic degradation processes have been active for a significant period and that bioavailable iron may be depleted. The unsaturated zone sample with less than 10% oil (Fig. 7A) has a significantly lower population than that with more oil (Fig. 7B), suggesting that the low oil location may be relatively carbon limited.

The existence of a large active methanogenic population in the oil-contaminated unsaturated zone is consistent with observations of Hult et al. [21], who documented methane gas production in the same area. Methanogenic activity in the unsaturated zone can have a substantial influence on the chemistry of the plume. Any recharge that enters the aquifer through the oil body will be high in organic acids and methane and have zero dissolved oxygen. The reason for the strongly methanogenic conditions in the unsaturated zone can be understood by examining the percentage of saturated pore space. Dillard et al. [11] characterized the oil and water saturation for a vertical profile through this location. They found a low permeability horizon at about 426 m above sea level, just below the elevation where the 10% oil saturation contour widens (Fig. 2). A combination of perched water and high oil content results in total fluid saturations of 70% for these two sample locations [11]. With such a high percentage of the pore space saturated, it is reasonable that there is little or no connection of the gas-filled pores to surface oxygen supplies.

Discussion

The combination of closely spaced vertical profiles and broadly spaced point samples provides a comprehensive picture of the distribution of physiologic types in the anoxic portion of the plume. Although dissolved methane concentrations of 12 mg/L are found over the entire anoxic portion of the plume [2], closely spaced samples were necessary to delineate the methanogenic zone. Several other workers have observed that redox conditions change rapidly in the vertical direction. Smith et al. [31] observed that peak denitrifying activity was confined to a vertical niche of 1 m over a 6 m thick anoxic zone of a sewage plume. Cherry [9] has proposed a conceptual model of plumes in which a high-concentration core of the plume, which is less than 1 m thick, is surrounded by a low-concentration fringe. If we envision the core of the plume as the zone that has progressed to methanogenesis at this site, then this zone extends only 0.25–1 m vertically (Fig. 3). Compared to the vertical profiles, the broadly spaced samples did a relatively poor job of delineating the methanogenic zone. The implication for field investigations in support of natural attenuation is that small spatial separation of samples is needed to delineate the methanogenic zone. The scale depends on the age of the plume and the overall vertical thickness. Thus, after 20 years, the center 50% of the laterally migrating plume below the oil has evolved to methanogenic conditions. Because methanogenic degradation of petroleum hydrocarbons ensues after dissolved oxygen and Fe(III) have been depleted, it is important to delineate the methanogenic zone of the plume. Models used to predict the long-term behavior of the plume should be based on the expansion rate of this methanogenic core.

The low suspended ratios for the majority of samples indicate that both in the plume and at the background site most of the population is attached to the sediments. Attached percentages ranging from 50 to 99% also have been observed at other contaminated sites (e.g., [17, 18]). Attachment may promote a number of advantages in ground water, including a predictable nutrient flux, access to solid-phase nutrients [29], protection from toxicity [5], and the association of interdependent anaerobic communities [14]. The portion of samples with less than 50% suspended have an average value of 15% (Fig. 5). The relatively constant ratio suggests that attached and suspended populations increase or decrease together. This is consistent with the results of Peyton et al. [28], who found that increased substrate flux resulted in increased growth and desorption of active organisms. Because 15% is a significant fraction of the population, models that attempt to maintain a mass balance for the total biomass need to account for both the suspended and the attached organisms. Previous laboratory studies with flow-through columns [28] and an intermediate-scale cell [25] showed that quantifying biomass in the effluent, in addition to attached biomass, was necessary to obtain a good mass balance.

One-fifth of the samples had suspended percentages greater than 50%, which is high compared to previous results [17, 18]. Eleven samples had suspended organisms, but

no attached ones of the same type. It is possible that these high values are an artifact of the method. The population numbers obtained for the water are usually consistent with the highest of those on sediments within the drained interval (Fig. 6). If water is drained from a large interval, the suspended populations found may reflect attached populations that exist anywhere within the drained interval. Thus, the ratio of suspended to attached microorganisms may be incorrect for that location. For the broadly spaced samples illustrated in Fig. 4, drained intervals could have spanned tens of centimeters, creating a situation in which suspended microorganisms are found but attached ones are not.

It is impressive that 10–100 times greater numbers of culturable methanogens, fermenters, sulfate-reducers, and denitrifiers were found in the unsaturated zone than in the saturated zone. Bone and Balkwill [6] documented a decrease in culturable numbers with depth in the unsaturated zone at a pristine site in Lula, Oklahoma. They attributed this trend to availability of an increased flux of nutrients from the surface at shallower depths. Ghiorse and Wilson [15] have suggested that nitrogen and phosphorus limitations are not likely in pristine aquifers, but may occur in contaminated aquifers with high dissolved organic carbon. This is consistent with the results of Rogers et al. [29], who found strong evidence for phosphate limitation in the saturated zone at the Bemidji site. Moreover, nitrate drops to below detection in the core of the plume [4]. These results indicate that, in modeling microbial growth at sites where organic contaminants act as growth substrates and are present in high concentrations, it may be important to account for the availability of additional nutrients besides carbon.

Summary and Conclusions

Using the MPN method we determined the population distributions for six physiologic types of microorganisms in the anaerobic portion of a petroleum hydrocarbon plume. Both suspended and attached numbers were estimated for aerobes, denitrifiers, iron-reducers, heterotrophic fermenters, sulfate-reducers, and methanogens. There were large differences between the pristine and contaminated locations at the site. For example, there were 10^2/g total methanogens in the plume and none detected in the background. The distribution of dominant physiologic types of microorganisms is consistent with the known geochemical evolution of the plume from iron-reducing to methanogenic conditions. There was a significant inverse correlation between numbers of methanogens and iron-reducers, indicating that the MPN data are capable of delineating evolving methanogenic conditions. However, culturable iron-reducers and methanogens were usually found together, suggesting that these two processes may be active simultaneously.

The methanogenic zone in the plume can be surprisingly narrow and was difficult to locate with samples spaced at meter intervals. A combination of closely spaced profiles and broadly spaced point samples provided a comprehensive picture of the spatial distribution of the populations. After 20 years of contamination, peak numbers of methanogens were found in two zones: in the area where nonaqueous oil is present and in the center 25–50% of the plume below the oil. Unsaturated zone locations contaminated with nonaqueous oil had 10–100 times greater numbers of methanogens than any location below the water table. Thus, methanogenic degradation is apparently occurring in the unsaturated zone near the residual nonaqueous oil. Moreover, the high culturable numbers in the unsaturated zone compared to the saturated zone suggest that access to macronutrients may limit microbial growth in the saturated zone at this site. Comparisons of suspended to attached numbers showed that an average of 15% of the total population is suspended, which is consistent with other reported values.

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